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ISOLATION, NUTRITION and METABOLISM of PHOTOSYNTHESIZING PLANT TISSUES

by

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FOREWORD

This is the final report on 5 years of work (June 1961 - June 1966) completed at the University of Wisconsin by Professor A. C. Hildebrandt and his associates under Contract No. DA 19-129-QM-1817 (N).

The initial objective of achieving photosynthesizing plant tissue cultures has not been attained, but many valuable studies of growth and nutrition of heterotrophic cultures have been carried out, and certain factors affecting development of chlorophyllous tissue have been evaluated. These studies have supported our in-house project on the development of plant tissue cultures as a potential food source.

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ABSTRACT

There appears a great potential in tissue cultures of higher plants as a means of producing an abundant supply of fresh, edible, tasty, nutritious plant food for gas exchange in difficult situations and in space travel. Chlorophyllous and nonchlorophyllous strains of edible plant tissues have already been established from many plant species. The requirements for growth and chlorophyll production are influenced by the composition of the medium on which they are grown and by other environmental factors, including light, temperature and acidity of the medium. Nitrate is an excellent source of nitrogen. Tissues grown in liquid media on a shaker or in aerated media tend to fragment into single cells and small clumps of cells. Tissues on agar media may be grown as undifferentiated masses of cells or may be induced to differentiate roots, stems, leaves and plants by modifying the nutrient and other environments. Under space conditions the chlorophyllous tissues would have unlimited sunlight as energy for photosynthesis, would utilize carbon dioxide, and would produce oxygen in the process of synchesizing carbohydrate for food. Such abilities for growth and differentiation as a single cell or as tissue masses and even plants suggest this method has a great built-in potential to select for almost any type of food quality desired.

Introduction

The idea of isolation, growth of cells, differentiation of cells into tissues, organs, and eventually even into whole plants in tissue culture has long stimulated research by biologists. Such investigations strike at understanding the bases of growth itself. They suggest methods of clarifying normal and diseased growth at the cellular level. The in vitro study of higher plant cells provides the means of studying many details of cellular growth and metabolism in higher plants with the same techniques and precision used for sterile cultures of microorganisms.

Attempts to culture higher plant cells received previous attention starting in 1902 by Haberlandt in Germany. Growth in vitro was tried of many types of plant cells and organs. Much of the early work was directed to growing isolated root and stem tips. Root tips of tomato were finally established in sterile culture in 1937. Many different nutrient media were developed in the process of learning how to culture the root tips for unlimited periods. This background information on nutrition made it possible to also culture callus of higher plants. True cell cultures of tobacco stem and carrot root callus were successful for the first time in 1939 in independent studies carried on in the United States and in France. The original root cultures established in 1937 and the callus cultures in 1939 are still growing today after many subcultures. The rate and type of cell growth are similar to that in the original cultures. It is now possible to isolate and grow higher plant cells from many plant species. These have been used to study a variety of problems of normal and diseased cell growth.

Cell cultures of higher plants also appear to have a high potential as a means of producing an abundant supply of fresh, edible nutritious and flavorful food for humans in difficult situations, as in space travel and also here on earth. In the process of photosynthesis, chlorophyllous tissues also remove carbon dioxide from the air and produce oxygen. The cultures grow in chemically defined liquid media or on agar media. Modifications of the culture environment may induce cultures to produce leaves, stems, roots or plants that simulate natural food. Other desirable qualities of foods are already available or may be developed by selection for their qualities. Large-scale productions in tank or continuous cultures seem practical with technology already available.

The purpose of this project has been to exploit tissue cultures of higher plants as a source of human food. Cultures were established from many green edible plant species. Considerable progress has been made to determine the nutritional and other environmental requirements for growth of the chlorophyllous, photosynthesizing strains of tissues that grow rapidly and contain the desirable qualities of food for human consumption. Such results are encouraging that even better cultures can be developed. Details of these studies are presented on the following pages.

Strains of higher plant cells established in tissue culture

Plant tissue cultures were attempted in this laboratory from 32 species of edible plants. Roots, stems, leaves, seeds and embryos were used as sources of the cell cultures. Many different liquid and solid media were tested. The compositions of several of the most useful basic media are given in Tables 1 and 2. Many modifications of these were tested and are described under specific parts of this report.

Table 1. Constitution of various nutrient media with T-medium serving as the basic medium (Hildebrandt, 1962).

1. T-medium

Na_2SO_4	800.0 mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	400.0
$MgSO_4 \cdot 7H_2O$	180.0
KNO_3	80.0
KCl	65.0
$NaH_2PO_4 \cdot H_2O$	33.0
$MnSO_4 \cdot 4H_2O$	0.45
$ZnSO_4 \cdot 7H_2O$	0.6
H_3BO_3	0.00375
KI	0.03
$Fe_2(C_4H_4O_6)_3$	40.0
Glycine	3.0
Thiamine·HCl	0.1
Sucrose	20000.0
Agar	6000.0

2. THS-medium

Complete T-medium plus the following:

KCl	845.0 mg/l
$NaNO_3$	1800.0
$NaH_2PO_4 \cdot H_2O$	300.0
$(NH_4)_2SO_4$	790.0
Kinetin	0.5
Myo-inositol	100.0
Calcium pantothenate	2.5

3. C-medium

Complete T-medium plus the following:

Coconut milk	150 ml/l
Calcium pantothenate	2.5 mg/l
Alpha-naphthaleneacetic acid	0.1

4. D-medium

Complete C-medium plus the following:

2,4-dichlorophenoxyacetic acid 6 mg/l

Table 2. Preparation* and composition of Murashige and Skoog's (1962) medium as used in the present experiments.

Stock soln.	Constituents	Conc. in stock soln. gm/l	Volume of stock soln. in final medium - ml/l	Final conc. in medium mg/l
A	NH_4NO_3	82.5	20	1650.0
B	KNO_3	95.0	20	1900.0
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34.00		170.0
	KI	0.166		0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.05		0.25
	$CoCl_2 \cdot 6H_2O$	0.005		0.025
D	$CaCl_2 \cdot 2H_2O$	88.0	5	440.0
E	$MgSO_4 \cdot 7H_2O$	74.0	5	370.0
	$MnSO_4 \cdot 4H_2O$	4.46		22.3
	$ZnSO_4 \cdot 7H_2O$	1.72		8.6
	$CuSO_4 \cdot 5H_2O$	0.005		0.025
F[#]	$Na_2 \cdot EDTA$	7.45	5	37.35
	$FeSO_4 \cdot 7H_2O$	5.57		27.85
G	Thiamine·HCl	0.02	5	0.1
	Nicotinic acid	0.1		0.5
	Pyridoxine·HCl	0.1		0.5
	Glycine	0.4		2.0

Addendum:
Sucrose - 30 gm/l, myo-inositol - 100 mg/l, indole-3-acetic acid - 10 mg/l, kinetin - 0.04 mg/l, agar - 10 gm/l.

* The stock solutions A-g were prepared and stored in a refrigerator (never more than 4-6 weeks) and mixed just before preparing the final medium.

[#] The $FeSO_4 \cdot 7H_2O$ is dissolved in ca 200 ml double distilled water. The $Na_2 \cdot EDTA$ is dissolved in ca 200 ml double distilled water, heated and mixed (under continuous stirring) with the $FeSO_4 \cdot 7H_2O$ solution. After cooling, the volume is adjusted to 1000 ml. Heating and stirring result in a more stable Fe-EDTA complex.

Callus cultures were attempted from stems and leaves of asparagus, beet, Brussels sprouts, cabbage, carrot, cauliflower, celery, cucumber, horse-radish, lettuce, parsley, pea, potato, rhubarb, spinach, Swiss chard and tomato. Callus cultures also were attempted from parts of aseptically grown seedlings or excised embryos of asparagus, barley, beet, carrot, cucumber, endive, Idaho refugee bean, lettuce, mustard, navy bean, oats, pea, red kidney bean, rice, rye, spinach, squash, sunflower, Swiss chard, tomato and wheat. Established callus cultures from tomato, grape, 9 varieties of rose and potato were included in the growth tests.

Isolations of tissues were attempted from roots, stems, and leaves. Cross sections of leaf petioles and stems were used as sources of callus. Embryos also were dissected out and grown in vitro as sources of callus or normally differentiated plants. The latter sterile plants were used as sources of sterile plant parts in order to obtain callus cultures.

The tissue and organ isolations were attempted after surface disinfection with a 15-60 sec dip in 95% ethyl alcohol followed by 1-10 min in a 1:10 dilution with water of commercial 6.4% sodium hypochlorite and 1-2 min in each of 7 changes of sterile distilled water.

The original plant parts used as sources of callus, after surface sterilization were aseptically planted on the T-, C- and D- media and incubated in the greenhouse with an 18-hour daylength, 24-hour daylength or in the dark. The media for growth of the cultures were adjusted to pH 5.7-5.9 with HCl or NaOH. Tissues in most cases grew best on media of acidities from pH 5.5-5.9 (Table 3). The optimum temperature for growth of most tissue strains is about 26°C (Table 4). Supplementary light was provided by GE power groove, Sylvania Gro Lux and 100-w incandescent lamps. The callus tissues that grew from the isolated plant parts were grown in continuous light at 26°C.

Embryos placed on T- or C-medium produced normal plants but on D-medium developed callus instead. Stem and root pieces grew little or none on T-medium. Plant parts of many species produced callus on C- or D-medium. Carrot, endive, grape, lettuce, parsley, rose, red kidney bean and navy bean gave excellent callus growth on C-medium. Parsley, grape, potato, rose, tomato and navy bean also grew well on D-medium. Endive tissue produced leafy-stems on the surface of the cultures.

Table 3. Influence of the acidity of the culture medium on chlorophyll formation and growth of callus tissue of edible plants

Name of tissue	Final pH of medium				Chlorophyll content
	5.0	5.5	6.0	6.5	
Carrot	1.21*	1.21	1.31	1.31	+++
Rose (PG)	1.03	1.26	1.35	1.28	++
Tomato (A6-6)	0.75	1.25	0.88	0.89	+
Rose (BL)	2.05	2.55	2.44	2.39	-
Red Kidney Bean	1.94	2.20	2.16	1.78	-
Endive	0.52	1.26	1.45	1.37	+
Lettuce leaf	0.42	0.81	1.13	1.07	++
Lettuce stem	0.60	0.99	0.91	1.00	++

*Average wet weight in grams of tissue after 4 weeks' growth.

Table 4. Growth in the dark of edible plant tissue with different temperatures

Name of Tissue	Temperature °C						
	36°	32°	28°	24°	20°	16°	12°
Carrot	0.23*	2.15	2.94	4.01	2.02	0.99	0.21
PG	1.34	1.86	1.79	1.72	1.52	1.33	0.91
A66	0.04	1.35	1.44	1.89	0.56	0.37	0.06
GP2sc216	1.49	2.09	2.50	2.48	0.61	0.34	0.15
BL	0.45	2.82	3.14	2.50	1.50	0.90	0.40
NB	0.06	0.95	0.54	1.55	0.91	0.72	0.08
LS	1.81	1.12	0.80	0.91	0.46	0.47	0.21
RKB	0.73	1.57	1.97	1.78	1.25	1.11	0.28
NT	0.09	0.17	1.57	1.61	0.66	0.52	0.21
Parsley	0.42	1.77	1.84	1.90	1.39	1.38	0.44
Endive	1.71	2.75	2.14	1.23	0.18	0.14	0.12
GS6sc18	2.49	3.17	3.11	2.50	1.50	0.80	0.26
LL	2.53	1.66	1.28	0.96	0.75	0.50	0.24
A (Potato)	0.07	0.34	1.05	1.14	0.71	0.59	0.32

*Each number is the average net weight in grams of 24 pieces from 6 replicate culture bottles. Original tissue pieces weighed 50 mgs.

Culture growth rates

The relative growth rates of some strains of tissue on basic media are seen in Tables 5, 6, 7, and 8. Growth rates of the tissues varied with the species and environment. Wet and dry weight measurements of many tissue strains at weekly intervals showed that the growth curves follow a sigmoid pattern typical of the growth of plants and microorganisms. The fresh and dry weights increased simultaneously. In many cases, with the exception of endive tissue, there was increasing accumulation of water and therefore a decreasing dry weight percentage with age. Endive tissue, however, had decreased water content with increasing age, resulting in a final high dry weight percentage.

During the callus tissue growth, there is a steady increase in the number of cells by cell division and cell size, resulting in increase in volume, fresh and dry weight. There was no evidence of any unusual increase in size of the cells with increase in age of the cultures. Growth results from synthesis of new protoplasmic and cell wall material and by increasing cell populations along with cell expansion.

Growth of various tissues was compared in liquid suspension cultures on rotary or reciprocating shakers. Most cultures grown in suspension are masses of cells and small colonies of cells. Dissociation of the liquid cultures into single cells varied from 5 to 50% depending on the species and strain of tissue. This material provides a wealth of potential as a source of fast growing, chlorophyllous, nutritious tissue with different tastes, textures and other desirable food qualities.

Table 5. Relative growth of isolated plant tissues *in vitro* on 3 media in the light

Source of callus	Medium* T + CH	C	D
Bean:			
Navy stem		++	+++
Red Kidney stem	+	++	+++
Refugee stem			+++
Carrot root	++	+++	+
Cucumber stem		+	
Endive embryo	++	++	+
Grape:			
Normal stem GS6SC 25 (single cell clone)		+++	+
Normal stem GX6SC 18 (single cell clone)		+++	
Phylloxera gall GP2SC 4 (single cell clone)	-	+	+++
Phylloxera gall GP2SC 216 (single cell clone)		+	+++
Lettuce leaf petiole		+++	
Lettuce stem	+	+++	
Parsley leaf petiole		+++	++
Pea stem	++	+	+
Potato:			
A tuber			++
Ag 231 tuber			+
'Katahdin' tuber			++
'Kennebec T^6' tuber			++
'Kennebec T^1' tuber			++
Rose:			
'Better Times' stem	+	++	+++
'Blaze' stem	+++	+++	+++
'Chrysler Imperial' stem	-	++	+++
'Helen Traubel' stem	-	++	+++
'Multiflora' stem			++
'Pink Garnett' stem	+	+++	+++
'Red Garnett' stem	++	++	+++
'Rose lucida' stem			+
Spinach leaf petiole		+	+
Tomato:			
Crown gall A6		++	+
Crown gall A6-6	+	+++	++
Normal embryo		++	++
Normal stem	+	++	+++

*T + CH = basal mineral salts-sucrose medium + casein hydrolysate (3 g/liter), NAA (0.1 mg/liter), i-inositol (300 mg/liter).

Growth: - = no growth; + = slight, ++ = moderate; and +++ = excellent growth. Blank spaces indicate the media were not tried.

Table 6. Growth of callus at 6 weekly intervals on C-medium. Each number is the average wet weight in mg of 24 tissue pieces.

Source of callus	Average wet weight in mg after days*						Chlorophyll content**
	7	14	21	28	35	42	
Bean:							
'Red Kidney' stem	577	684	949	1052	1093	1322	-
Carrot root	535	929	1291	1968	2180	2239	+++
Endive embryo	357	685	968	1297	1745	2010	++
Grape Phylloxera gall SC 216	133	188	367	406	638	940	-
Lettuce stem	331	508	1013	1373	2210	2328	+
Potato:							
'Kennebec' tuber	151	206	278	289	273	243	+
Rose:							
'Blaze' stem	433	612	1429	1744	2773	2654	-
'Pink Garnett' stem	305	597	812	1123	1328	1397	++
Tomato:							
stem	210	439	481	897	786	1024	-
A_{6-6} crown gal	438	610	1067	1135	1183	1419	+

* The tissue pieces at the start weighed in mg: Red Kidney bean (250), carrot (250), endive (150), grape (100), lettuce (225), potato (125), rose var. 'Blaze' (225), rose var. 'Pink Garnett' (150), tomato stem (125), tomato A_{6-6} crown gall (225).
** Chlorophyll content rated from: - (none visible) to +++ (strong).

Table 7. Growth of callus at 6 weekly intervals on basal T-medium containing 2% sucrose plus alpha-naphthaleneacetic acid. Each number the average wet weight in mg of 24 tissue pieces.

Source of callus	Average wet weight in mg after days*						Chlorophyll content**
	7	14	21	28	35	42	
Bean							
'Red Kidney' stem	229	187	142	139	135	131	-
Carrot root	438	657	781	1130	1400	1480	++
Endive embryo	395	451	441	436	423	400	-
Grape Phylloxera gall SC 216	144	149	201	154	132	135	-
Lettuce stem	331	434	371	528	591	545	-
Potato:							
'Kennebec' tuber	167	247	155	243	262	244	-
Rose:							
'Blaze' stem	393	448	382	429	495	571	-
'Pink Garnett' stem	318	442	431	384	397	423	-
Tomato:							
stem	367	409	562	821	769	731	-
A_{6-6} crown gall	709	957	1121	1438	1533	1708	-

* The tissue pieces at the start weighed in mg: Red Kidney bean (100), carrot (225), endive (250), grape (125), lettuce (150), potato (125), rose var. 'Blaze' (225), rose var. 'Pink Garnett' (125), tomato stem (200), and tomato A_{6-6} crown gall (250).
** Chlorophyll content rated from: - (none visible) to +++ (strong).

Table 8. Growth of callus at 6 weekly intervals on the basal T-medium without sucrose. Each number the average wet weight in mg of 24 tissue pieces.

Source of callus	Average wet weight in mg after days*						Chlorophyll content**
	7	14	21	28	35	42	
Bean:							
'Red Kidney' stem	143	108	128	124	136	113	-
Carrot stem	528	634	712	719	760	765	+++
Endive embryo	179	187	195	209	193	191	-
Grape:							
Phylloxera gall SC216	117	108	100	82	85	75	-
Lettuce stem	292	260	228	246	252	267	-
Potato:							
'Kennebec' tuber	174	157	143	162	144	144	-
Rose:							
'Blaze' stem	413	436	407	400	363	335	-
'Pink Garnett' stem	181	164	138	131	136	154	-
Tomato:							
Normal stem	302	265	256	245	236	225	-
A_{6-6} crown gall	579	515	439	412	410	399	-

* The seed tissue pieces at the start weighed in mg: Red Kidney bean (100), carrot (225), endive (125), grape (100), lettuce (150), potato (100), rose var. 'Blaze' (225), rose var. 'Pink Garnett' (125), tomato stem (200), and tomato A_{6-6} crown gall (250). The data start after the 1st week when the sugar in the transfer apparently has been used.

** Chlorophyll content rated from: - (none visible) to +++ (strong) after 6-week growth period.

Effects of concentrations of various sources of carbon on tissue growth

The growth of the tissues was tested on various media with different sources of carbon, with or without coconut milk (CM). The effect of different concentrations of CM in T-medium on tissue growth is seen in Table 9. Tomato, lettuce, parsley, navy bean, red kidney bean, endive, grape, rose and carrot tissues grew progressively better with increased CM through the range 0-16%.

Tissue growth and chlorophyll formation were also tested on media with different concentrations of sugars on media with reduced (0.25%) CM. Coconut milk provided sufficient carbohydrate to sustain growth of the tissues at high concentrations. At the same time, tissues on media without coconut milk grew little or none, or died, while on 0.25 per cent they remained alive but did not grow in weight appreciably.

In order to test the relative availability of various sugars and the concentration effects for growth, the tissues of a number of species were, therefore, grown on C-medium with only 0.25 per cent coconut milk. The wet weights of tissues after 6 weeks growth on sucrose, maltose, dextrose and levulose concentrations are summarized in Table 10. Experiments with other species and sugars are in progress.

Table 9. Effect of coconut milk supplements to the basal T-medium on growth of plant tissue cultures. Growth measured as wet weight in grams after 6 weeks growth

Tissue	Coconut milk concentration					
	0	0.25	1.0	4.0	15.0	16.0
Endive	.62	.63	.82	1.52	3.12	
Grape	.25	.26	.32	.85		2.49
Carrot	61	.60	.62	.98	1.97	
Rose, pink garnett	.31	.32	.35	.49		1.50
Rose, better times	.26	.27	.29	.31		.61
Red kidney bean	.20	.21	.25	.41		1.21

Table 10. Effect of sugar and concentration on tissue growth and chlorophyll formation on C-medium with 0.25 per cent coconut milk.

Tissue	Sugar	sugar concentration (per cent)*				
		0	0.25	1.0	4.0	8.0
Carrot root	sucrose	.2++	.3++	.5+++	.6+++	.3++
	maltose	.5++	1.0+++	1.0+++	.7++	.5++
	dextrose	.3+	.7++	.7+++	.6++	.2+
	levulose	.4+++	.6+++	.4+	.2-	.1-
Grape stem (Sc18)	sucrose	.2-	.7-	.9-	.7-	.4-
	maltose	.2-	.6-	.7-	.6-	.4-
	dextrose	.1-	.6-	.5-	.4-	.1-
Lettuce stem	sucrose	.5+	.7+	.8-	.6-	.4-
	maltose	.3+	.4+	.4-	.3-	.2-
Parsley stem	sucrose	.2-	.5-	.5-	.4-	.3-
	maltose	.09-	.4-	.4-	.3-	.2-
	dextrose	.2-	.5-	.6-	.4-	.2-
Rose (Blaze) stem	sucrose	.2-	.6-	2.1-	2.0-	1.1-
	maltose	.2-	.7-	1.0-	1.1-	1.0-
	dextrose	.2-	.6-	1.8-	1.7-	1.0-
	levulose	.1-	.6-	1.8-	1.8-	.1-
Rose (P. G.) stem	sucrose	.2-	.3-	.3-	.3-	.2-
	maltose	.1-	.3-	.3-	.3-	.3-
	dextrose	.07-	.3-	.3-	.3-	.2-
Tomato stem	sucrose	.1-	.3-	.5-	.4-	.2-
	maltose	.1-	.3-	.3-	.3-	.2-
	dextrose	.07-	.2-	.3-	.3-	.1-
Tomato gall	sucrose	.05-	.3-	.9-	.5-	.2-
	maltose	.2-	.5-	1.0-	1.0-	.6-
	dextrose	.2-	.5-	1.0-	.6-	.2-
	levulose	.1-	.4-	.4-	.1-	.1-
Endive	dextrose	.2+	.2-	.2-	.1-	.1-

*Wet wt. of tissues given in gms. after 6 wks growth. Chlorophyll content rated from - (none visible) to +++ (strong).

The optimum concentration of sugar for growth was usually between 0.5 and 3%. However, lower and higher concentrations often reduced growth but resulted in increased chlorophyll contents. This suggested that when the tissues are forced by unfavorable lack of sugar they are induced to synthesize more of their carbohydrate needs through photosynthesis. This is again encouraging because by modifying the nutrients in the medium we may learn more about the requirements for securing chlorophyllous cells capable of active, autotrophic growth.

Carrot, lettuce leaf and stem and tomato tissue grew very well on MS-medium with even 0.5% sucrose. On this medium, in the complete absence of sucrose in the medium, the rate of growth are much reduced. The substitution of soluble starch for sucrose in the medium inhibited growth. The capacity of these tissues for autotrophic growth in a synthetic medium is still under investigation. Many of these tissues will already grow for several transfers on C- or THS-media without added sucrose.

Growth of tissues on different nitrogen sources

Previous studies showed that most tissues favored nitrate as a nitrogen source. In fact, earlier studies with many species showed the concentrations of nitrogen used in the basic T-medium were about optimum. Studies were, however, made to determine the optimum concentration of nitrate for several of these edible plant tissues and the results are summarized in Table 11. These results again indicate the concentration of nitrate used in the basal media are satisfactory for excellent growth of the tissues.

Urea was also tested as a nitrogen source. The relative growth rates and chlorophyll contents of the tissues on different concentrations are shown in Table 12.

Similar growth data are presented in Table 13 for results with ammonium nitrate and the nitrogen source.

Table 11. Influence of different $NaNO_3$ concentrations on growth and chlorophyll production of edible plant tissue

Name of Tissue	Medium*	Concentration $NaNO_3$ in gm/l					Growth period in days
		0.45	0.9	1.8**	3.6	7.2	
		mg***	mg	mg	mg	mg	
Carrot root	T-N	697	888	677	700	373	21
		++	+++	+++	+++	+++	
	T+I	697	826	788	695	294	21
Lettuce stem	T-N	328	428	320	260	173	29
		0	0	0	0	0	
	T+I	643	623	527	471	372	28
Parsley leaf	T-N	680	670	592	565	370	29
		+	+	+	+	0	
	T+I	510	571	596	465	344	29
		+	+	+	+	0	
	T+I+D	221	228	221	154	138	29
	T+D	264	248	249	(168)	154	29
PG rose stem	T-N	126	130	115	122	83	33
		0	0	0	0	0	0
	T+I	332	390	390	242	147	32
		++	++	++	++	+	
BL rose stem	T-N	136	170	123	93	77	32
		0	0	0	0	0	
	T+I	219	263	244	163	105	29
		C	0	0	0	C	
	T+I+D	941	913	868	443	120	28
		0	0	0	0	0	
	T+D	2083	2416	2401	2086	195	28
Grape stem GS6sc18	T-N	767	796	656	615	195	32
		+	+	+	+	+	
	T+I	1011	974	859	660	202	32
		++	++	++	++	+	
Red kidney bean	T-N	242	243	291	201	109	33
		0	0	0	0	0	
	T+I	42	59	65	63	58	30
	T+I+D	69	56	71	74	62	30
	T+D	159	179	301	462	195	33
Tomato gall A66	T-N	381	373	364	242	118	30
		+	+	+	+	0	
	T+I	523	515	602	466	249	30
		+	+	+	+	+	
Navy bean	T-N	402	358	409	130	91	30
	T+I	63	77	126	64	70	31
	T+I+D	111	156	84	121	77	31
	T+D	77	83	71	62	84	33
Lettuce leaf	T-N	1426	1133	1062	907	609	28
	T+I	1199	1328	1159	844	633	28
Tomato stem	T+I+D	282	256	258	211	183	22
	T+D	504	492	427	381	284	32
Endive embryo	T-N	612	599	543	487	244	22
	T+I	881	1133	967	790	425	22

*Media: "T-N" = Tobacco medium + high salts - NO_3. Added $NaNO_3$ in concentrations of: 0.45 - 0.9 - 1.8 - 3.6 and 7.2 grams per L medium. "T+I" = T-N + NAA $1x10^{-4}$ (1 ml/L medium). "T+I+D" + T-N + NAA $1x10^{-4}$ (1 ml/L medium) + 2,4-D 6 mg/L medium. "T+D" = T-N + 2,4-D 6 mg/L medium.

**1.8 gm/l = the NO_3 concentration in high salts medium.

***Each number the average wet weight in mg. 24 tissue pieces from 6 cultures. Original tissue piece 50 mgs. Chlorophyll rated from 0 = none; + = poor; ++ = fair; +++ = excellent.

Table 12. Influence of different concentrations of urea as a nitrogen on growth and chlorophyll content of edible plant tissue

Name of Tissue	Medium*	Average wet weight and chlorophyll content of tissues on 4 media with urea added or nitrogen source (gm/l)** .001	.01	0.1	1.0	10.0	Control C or D-*** medium
Carrot Root	T	236	225	458	221	146	2202
	T+NAA	199	209	291	206	156	+++
	T+NAA+D	172	184	245	250	154	
	T+D	194	206	279	211	158	
		+	+	+	+	+	
PG Rose Stem	T	115	131	136	73	64	2262
	T+NAA	325	362	264	83	88	++
	T+NAA+D	264	369	376	83	76	
	T+D	216	260	263	102	106	
		+	+	+	-	-	
Tomato Gall A6-6	T	193	257	304	116	100	2387
	T+NAA	333	279	604	357	96	+
	T+NAA+D	263	344	842	1757	75	
	T+D	151	197	373	77	54	
		+	+	+	-	-	
Grape Gall GP_2SC216	T	463	690	688	143	172	3110
	T+NAA	354	393	267	154	119	-
	T+NAA+D	159	311	203	132	111	
	T+D	365	427	313	130	129	
		-	-	-	-	-	
BL Rose Stem	T	291	317	646	63	70	4663
	T+NAA	183	290	718	99	84	-
	T+NAA+D	254	441	956	70	71	
	T+D	199	351	761	68	55	
		-	-	-	-	-	
Navy Bean	T	100	105	129	112	104	2615
	T+NAA	105	110	133	104	86	-
	T+NAA+D	107	120	114	109	92	
	T+D	103	107	131	106	99	
		-	-	-	-	-	
Lettuce Stem	T	228	357	526	106	84	2370
	T+NAA						+
	T+NAA+D						
	T+D	104	177	251	104	84	
		-	-	-	-	-	
Red Kidney Bean	T	102	106	136	104	98	3661
	T+NAA	173	194	559	112	94	-
	T+NAA+D	83	156	161	126	90	
	T+D	133	146	454	64	69	
		-	-	-	-	-	

Table 12 (cont.)

Name of Tissue	Medium*	Average wet weight and chlorophyll content of tissues on 4 media with urea added or nitrogen source (gm/l)** .001	.01	0.1	1.0	10.0	Control C or D-medium***
Tomato Stem	T	144	215	427	82	61	2137
	T+NAA	271	343	626	225	106	+
	T+NAA+D	190	393	725	279	65	
	T+D	144	287	456	205	94	
		+	+	+?	-	-	
Parsley Stem	T						
	T+NAA						
	T+NAA+D	147	177	295	152	111	1596
	T+D						+
		±	±	±	-	-	
Grape Stem GS6SC18	T	549	704	1332	150	126	2290
	T+NAA	513	662	1090	173	134	±
	T+NAA+D	238	280	185	127	109	
	T+D	318	349	379	151	138	
		-	-	-	-	-	
Lettuce Leaf	T	331	431	653	99	95	1904
	T+NAA						++
	T+NAA+D	165	172	199	187	109	
	T+D	220	202	329	123	102	
		-	-	-	-	-	

*Media: T = Tobacco medium + high salts-nitrate. Added urea in concentrations of: .001, 0.01, 0.1, 1.0 and 10.0 gm/l medium,
NAA = alpha-naphthaleneacetic acid ($1x10^{-4}$gm/l); D = 2,4-dichlorophenoxyacetic acid (2,4-D) (6 mg/l).
**Average wet weight of 24 tissue pieces. Original seed transfer pieces weighed 50 mg.
***Control tissues grown on C-medium or D-medium.

Generally the tissues grew less well on media with urea as a nitrogen source then they did on the control C- or D-media containing coconut milk or coconut milk plus 2,4-dichlorophenoxyacetic acid (2,4-D). The grape stem tissue at the median concentrations grew better than other tissues on urea. The amount of chlorophyll produced varied with the species of tissue and the concentration of urea used.

Ammonium nitrate was a nitrogen source. This compound in the synthetic media was used at .45, .90, 1.8, 3.6 and 7.2 gm/l. The average wet weights of the tissues starting from 50 mg pieces and the relative amounts of chlorophyll produced are seen in Table 13.

Table 13. The influence of different concentrations of ammonium nitrate on growth and chlorophyll production of edible plant tissues

Name of Tissue	Medium*	Average wet weight in mg and chlorophyll content of tissues grown on media with ammonium nitrate concentrations** .45	.90	1.8	3.6	7.2	Control C- or D-medium***
Carrot Stem	T+NAA	616 ++	663 +++	429 +++	186 ++	141 +	2202 +++
PG Rose Stem	T+D	120 0	118 0	112 0	102 0	77 0	2262 ++
Tomato Gall A6-6	T+NAA	644 0	955 +	1365 +	698 ++?	178 ++?	2387 +
Grape Gall GP2SC216	T	313 0	367 0	360 0	321 0	97 0	3110 -
Navy Bean Stem	T	95 0	131 0	112 0	98 0	111 0	2478 0
Lettuce Stem	T	166 0	205 0	175 0	148 0	100 0	2370 +
Red Kidney Bean Stem	T	125	241	310	224	175	2515 0
Tomato Stem	T+D	855 0	1138 0	873 0	679 0	230 0	2137 -
Parsley Stem	T+NAA	180 0	208 0	278 0	230 0	183 0	1596 +
Grape Stem GS63C18	T+NAA	294 0	235 0	207 0	155 0	81 0	2290 -
Lettuce Leaf	T	226	191	187	133	124	1904 ++

*Media: T = Tobacco medium + high salts-nitrate. Added NH_4NO_3 in concentrations of: 0.45, 0.9, 1.8, 3.6, 7.2 gm/l medium. NAA = alpha-naphthaleneacetic acid ($1x10^{-4}$gm/l); D = 2,4-dichlorophenoxyacetic acid (6 mg/l).

**Average wet weight of 24 tissue pieces. Original seed transfer pieces weighed 50 mg.

***Control tissues grown on C-medium or D-medium.

Growth of the tissues with ammonium nitrate as the nitrogen source was poor to good depending on the tissue strain, the concentration of ammonium nitrate used and the basic medium employed. These are preliminary results obtained to date. Carrot and tomato gall tissues produced the best amounts of chlorophyll with ammonium nitrate present.

Production of pigments in the tissue cultures

The composition of the medium and light influence pigment formation in certain strains of tissue. Little or no pigmentation was found in cultures grown in total darkness or in low light intensity. Increased day length and light intensity induced pigment formation to varying degrees depending on the species and medium on which the tissue was grown. Rose, parsley, grape and potato tissues developed red pigmentation in the light.

Chlorophyll developed in certain cultures grown in partial or continuous light. The D-medium inhibited chlorophyll production. Callus of pea, potato, tomato, and rose produced some chlorophyll however on D-medium Chlorophyll production was seen in callus cultures from carrot, endive, grape, lettuce, pea, potato and rose on C-medium. The best chlorophyll production was seen in cultures from carrot, endive, lettuce, pea, spinach, parsley, rose and tomato and these strains of tissue have been most extensively tested in this laboratory.

Growth of chlorophyllous tissue on basic media

Growth of chlorophyllous tissues was studied on T-, THS-, MS-, C- and D-media and various modifications of these media. Fresh and dry weights, dry weight/100 gm fresh weight (dry weight percentage of DW%) and optical density (OD) of the chlorophyll extracts of the tissues were determined after a 5-week growth period (Table 14). C-medium proved the best as far as increase in fresh weight is concerned. D-medium inhibited growth as compared to C-medium, except for parsley tissue. Synthetic media produced less vigorous growth than the C-medium, but the growth was satisfactory. MS-medium gave favorable results in all tissues except rose tissue. Since coconut milk is a very complex substance and its exact composition is not known, it was decided to conduct most of the experiments with defined media like the MS-, THS- and T-medium.

In order to ascertain whether the growth in various media was strictly a result of the composition of the medium or because of carried-over effects of the C- or the D-medium in stock cultures, the tissues were maintained for four transfers on each medium separately. Results of this experiment were that carrot, lettuce leaf and stem, parsley, rose and tomato tissues showed greatly reduced growth with each subsequent transfer on T- or THS-medium; endive tissue showed a slight increase or no marked change in growth rate. On C-medium all tissues except parsley either showed a steady rate of growth or an increased rate of growth. On D-medium most tissues maintained their rate of growth except carrot and endive which showed greatly reduced growth after each transfer. Continued maintenance on MS-medium improved growth in carrot, endive, lettuce stem and tomato tissues; parsley and lettuce leaf tissue showed reduced growth in subsequent transfers while the growth of rose tissue was completely inhibited.

There is some evidence of the acclimatization of the various tissues to different media, particularly the C-, D- and MS-medium, as is evidenced by increased growth in each subsequent transfer to the same medium.

Table 14 Fresh weight (FW), dry weight (DW), dry weight per 100 gms (DW%) and optical density (OD) at 665 mμ of various chlorophyllous tissues grown for 5 weeks in continuous light (100 ft. c.) at 25-26°

Tissue	Data	MEDIA				
		T	THS	C	D	MS
Carrot	FW	2.248	2.221	2.462	1.102	2.224
	DW	0.182	0.152	0.194	0.119	0.186
	DW%	8.11	6.85	7.87	10.77	8.36
	OD	0.27	0.20	0.30	0.30	0.19
Endive	FW	0.983	1.626	6.473	3.874	3.394
	DW	0.093	0.110	0.737	0.336	0.369
	DW%	9.41	6.78	9.86	8.68	10.88
	OD	0.17	0.32	0.23	0.10	0.24
Lettuce leaf	FW	5.417	2.074	11.080	0.676	5.645
	DW	0.279	0.127	0.631	0.062	0.312
	DW%	5.16	6.13	5.69	9.11	5.53
	OD	0.16	0.12	0.10	0.02	0.13
Lettuce stem	FW	2.150	1.774	6.556	0.757	3.324
	DW	0.119	0.097	0.388	0.058	0.239
	DW%	5.51	5.47	5.91	7.60	6.20
	OD	0.11	0.12	0.12	0.06	0.09
Parsley	FW	3.999	1.656	6.741	7.063	3.278
	DW	0.208	0.108	0.299	0.172	0.235
	DW%	5.19	6.54	4.43	2.43	7.17
	OD	0.12	0.12	0.09	0.08	0.08
Rose	FW	0.918	0.718	6.890	6.002	0.783
	DW	0.048	0.040	0.358	0.329	0.052
	DW%	5.17	5.57	5.20	5.49	6.62
	OD	0.02	0.01	0.03	0.10	0.05
Tomato	FW	2.274	3.433	6.740	4.873	2.543
	DW	0.128	0.192	0.344	0.279	0.180
	DW%	5.63	5.40	5 09	5.74	7.09
	OD	0.04	0.11	0.14	0.10	0.09

Iron nutrition for growth and chlorophyll production

Iron plays a basic role in plants in the biosynthesis of chlorophyll and in photosynthetic mechanisms and therefore comparisons were made of iron sources for growth and chlorophyll formation in the callus cultures. Five strains of callus were used: carrot tissue with a relatively high chlorophyll content; endive and lettuce with medium amounts; and tomato and parsley tissue with low chlorophyll contents. Two iron systems were substituted for the iron tartrate in the basic C- and D-media. Citric acid-ferrous sulfate, and ferric sulfate - EDTA complexes were used and the results are summarized in Table 15. An increase in the available iron did not markedly stimulate growth or chlorophyll

synthesis. Most chlorophyll occurred at the iron concentration optimum for growth. Visually and spectrophotometrically determined chlorophyll contents seen in Table 15 were related as follows per gram of tissue: +++, more than 12 alpha chlorophyll; ++, 8-12 alpha; +, 3-8 alpha, -, 0-3 alpha. The approximate ranges in chlorophyll contents of the five tissue strains were as follows: carrot root tissue (15-25 alpha/g); endive (13-24 alpha/g); lettuce (3-7 alpha/g); parsley (2-7 alpha/g); and tomato (0-3 alpha/g). For comparison the normal tomato plant stem and lettuce plant stem and leaf petiole contained 40-50 alpha/chlorophyll/g.

Table 15. Chlorophyll estimation of carrot, parsley, endive, tomato and lettuce tissues cultured on C- and D-media with Fe-citric acid and Fe-EDTA combinations in different concentrations.

	Concentration Fe-citric acid*						
Tissue	0	1.25	2.5	5.0	10.0	20.0	C/D
Carrot	++	+++	+++	+++	+++	++	+++
Parsley	-	+	+	+	+		+
Endive	-	++	++	+++	+++		+++
Tomato	-	-	-	-	-	-	-
Lettuce	+	++	++	++	+	+	++

	Concentration Fe-EDTA*						
	0	3	6	12	18	24	C/D
Carrot	++	+++	+++	+++	+++	+++	+++
Parsley	-	-	+	+	+	+	+
Endive	-	+	+	+++	+++	+++	+++
Tomato	-	-	-	-	-	-	-
Lettuce	-	+	+	++	++	++	++

*Concentrations are given in ml stock solution per 1 media. Chlorophyll content was visually estimated as follows: +++ strong chlorophyll, tissues green; ++ tissues light green; + poor chlorophyll, tissues slightly green; - no visible chlorophyll, tissues white, yellow or brown.

Effect of increased levels of iron and magnesium

Magnesium and iron particularly, as well as copper, potassium and ammonium salts, play important roles in the synthesis and/or metabolism of chlorophyll in plants. With this view in mind, the basic MS-medium was further modified by the addition of extra amounts of copper sulphate, potassium nitrate and monobasic ammonium phosphate. Increased concentrations of iron (Na_2, EDTA and ferrous sulphate) or magnesium sulphate) were added to the modified MS-medium (MMS). The MMS-medium was superior to the MS-medium for growth of carrot, endive, tomato and parsley tissue but regarded growth of lettuce tissue (Table 16). Addition of increased amounts of iron and magnesium caused marked inhibition of growth in a majority of tissues. Apparently, therefore, the level of iron and magnesium was at an optimal level in the MS-medium. No significant increase in chlorophyll development was seen in MMS-medium with or without the addition of higher concentrations of iron and magnesium.

Table 16. Growth of chlorophyllous callus tissues in Murashige and Skoog (MS) medium and in modified MS medium (MMS) with increased concentrations of iron (Na_2.EDTA and $FeSO_4 \cdot 7H_2O$) and magnesium ($MgSO_4 \cdot 7H_2O$). Cultures incubated at 26±2°C in continuous light (100-200 f.c.) on agar media for 5 weeks. MS medium has 37.35 mg/l of Na_2.EDTA and 27.85 mg/l of $FeSO_4.7H_2O$. Results are average of 32 pieces from 8 bottles.

	MEDIA									
Tissue	MS	MMS	MMS + Fe1	MMS + Fe2	MMS + Fe3	MMS + Fe4	MMS + Mg1	MMS + Mg2	MMS + Mg3	MMS + Mg4
Lettuce (Stem)	3.824	2.223	1.978	2.076	2.073	1.713	2.135	1.728	2.369	2.491
Lettuce (Leaf)	5.645	2.249	2.192	2.327	2.153	1.901	2.371	2.875	2.325	2.260
Carrot (Root)	2.224	6.340	2.445	3.157	2.139	3.177	4.542	4.227	3.971	4.027
Endive (Embryo)	3.394	3.863	2.335	1.615	0.818	2.220	0.909	2.996	3.215	3.340
Tomato (Crown gall)	2.543	8.220	8.220	3.8[illegible]	1.767	3.075	4.635	4.850	5.014	5.338
Rose (Stem)	0.745	0.783	0.985	0.80[illegible]	0.793	0.845	0.730	0.613	0.888	0.785
Parsley (Petiole)	3.278	3.830	2.160	1.158	0.928	1.787	2.295	1.353	1.589	0.755

MMS - modified MS (normal MS plus extra $CuSO_4.5H_2O$, 15 mg/l; KNO_3, 15 mg/l and $NH_4H_2PO_4$, 10 mg/l)
Fe1 - 56.02 mg/l Na_2.EDTA and 41.77 mg/l $FeSO_4 \cdot 7H_2O$
Fe2 - 74.7 mg/l Na_2.EDTA and 55.7 mg/l $FeSO_4 \cdot 7H_2O$
Fe3 - 112.05 mg/l Na_2.EDTA and 83.55 mg/l $FeSO_4 \cdot 7H_2O$
Fe4 - 149.4 mg/l Na_2.EDTA and 111.3 mg/l $FeSO_4 \cdot 7H_2O$
Mg1 - 50 mg/l $MgSO_4 \cdot 7H_2O$
Mg2 - 100 mg/l $MgSO_4 \cdot 7H_2O$
Mg3 - 150 mg/l $MgSO_4 \cdot 7H_2O$
Mg4 - 200 mg/l $MgSO_4 \cdot 7H_2O$

Growth effects on tissues of supplements to synthetic media

The synthetic media are most desirable for defining the effects of critical concentrations of various compounds on growth of the tissues. Some comparisons of growth on unusual synthetic media are shown in Table 17. The data show that good growth of many edible plant cultures is possible on synthetic media. Growth, chlorophyll production and differentiation may be controlled by selecting the proper medium for the particular tissue. Unfortunately, particular synthetic media are not always best for growth of all tissues, indicating there is still much room for improvement in media. Therefore, efforts have been made to determine better nutrient balances for growth of the tissues.

Table 17. Comparative growth of various chlorophyllous callus tissues in synthetic media. All tissues grown for 5 in continuous light (100-200 f.c.) at 26±2°C in agar media. Figures are fresh weight of tissue in gms/bottle from average of 32 pieces in 8 bottles.

	TISSUES					
Media	CRT	EE	PAR	A6-6	LS	LL
MS	2.224	3.394	3.278	2.54_	3.824	5.645
MMS	6.340	3.863	3.830	8.220	2.223	2.249
THS	2.221	1.626	1.656	3.433	1.774	2.074
THS + 125 mg/l CH	3.185	5.945	1.680	3.725	1.925	3.840
THS + 125 mg/l YE	4.322	10.240	--	--	2.078	6.275
MS-A-IEK	4.425	8.542	2.990	0.880	1.620	8.573
THS-IEK	3.740	8.798	1.676	1.215	1.628	6.760

MS - standard Murashige and Skoog medium; MMS - MS + $CuSO_4 \cdot 5H_2O$, 15 mg/l; KNO_3, 15 mg/l and $NH_4H_2PO_4$, 10 mg/l. THS - tobacco high salts medium. CH - casein hydrolysate. YE - yeast extract. MS-AK - MS medium without IAA and kinetin. MS-A-IEK - MS medium without IAA but with 200 mg/l inositol, 0.5 mg/l kinetin and 1000 mg/l edamin. THS-IEK - THS medium with 200 mg/l inositol, 0.5 mg/l kinetin and 1000 mg/l edamin.

Supplements of casein hydrolysate (CH) or yeast extract (YE) in the MS-medium were very inhibitory for growth of all tissues. The same supplements, however, added to the THS-medium resulted in greatly increased amounts of growth and chlorophyll formation in carrot, endive, and lettuce leaf tissues (Table 18).

Table 18. Growth of chlorophyllous callus tissues in Murashige and Skoog (MS) and tobacco high salts (THS) media supplemented with casein hydrolysate (CH) and yeast extract (YE), in MS medium without IAA and kinetin (MS-AK) and in MS and THS media without IAA but supplemented with 200 mg/l inositol, 0.5 mg/l kinetin and 1000 mg/l edamin (CH) (MS-A-IEK and THS-IEK). All tissues grown at 25±2°C in continuous light (200 f.c.) for 5 weeks. Figures are fresh weight in gms/bottle from average of 32 pieces in 8 bottles.

Medium	TISSUES					
	CRT	EE	PAR	A6-6	LS	LL
MS- Control	2.224	3.395	3.278	2.543	3.824	5.645
MS + CH in mg/l						
125	0.880	0.709	1.297	0.799	0.940	1.249
250	0.955	0.818	1.355	0.808	1.075	1.231
500	0.955	0.874	1.423	0.866	0.910	1.257
1000	1.075	0.832	0.817	0.920	0.810	1.171
MS + YE in mg/l						
125	no growth	no growth	no growth	no growth	no growth	no growth
500						
1000						
5000						
THS-Control	2.221	1.626	1.656	3.433	1.774	2.074
THS + CH in mg/l						
125	3.185	5.945*	1.680	3.725	1.925	3.840
250	3.575	5.975	2.110	1.690	1.875	2.345
500	2.360	5.415	1.770	1.520	1.490	1.785
1000	2.135	5.675	1.690	1.210	1.340	0.890
THS + YE in mg/l						
125	4.322	10.240**	no growth	no growth	2.078	6.275
500	2.565	9.070			1.658	6.975
1000	2.650	4.208			1.428	7.498
5000	1.320	2.425			0.478	--
MS-AK	2.413	3.575	1.035	1.170	no growth	no growth
MS-A-IEK	4.425	8.542#	2.990	0.880	1.620	8.573
THS-IEK	3.740	8.79?##	1.676	1.215	1.628	6.760

*Optical density (O.D.) of chlorophyll extract, 0.19. O.D. of chlorophyll extract from mature endive leaves, 0.6; O.D. of chlorophyll extract from young endive leaves, 0.37; **Optical density, 0.38.
#Optical density, 0.27; ##O.D., 0.28.

Best growth of endive tissue was achieved in THS-medium with 125 mg/l of yeast extract and the amount of chlorophyll produced in this medium was equal to the chlorophyll present in the inner, young and pale green leaves or about 60 percent of chlorophyll present in the outer, older and mature leaves in a rosette of endive grown in nature. Perhaps no other callus tissues grown in vitro have produced such high amounts of chlorophyll except some strains of tobacco tissue grown in this laboratory.

Indole-acetic acid and kinetin were essential in the MS-medium for the growth of most tissues except carrot and endive. Addition of increased amounts of inositol (200 mg/l as compared to 100 mg/l), kinetin (0.5 mg/l as compared to 0.04 mg/l) and edamin (100 mg/l; pancreatic digest of casein) supported excellent growth even in the absence of any exogenous supply of auxin in carrot, endive and lettuce leaf tissues.

Most of the tissues, including endive and carrot, grown in synthetic media had a normal and agreeable taste and were quite palatable. Endive tissue seemed particularly satisfactory as a source of human food. This tissue is bright green in color, juicy and crisp. It was induced to differentiate into innumerable tiny rosettes of leaves and shoots. The presence of a mild aromatic flavor, similar to that of natural endive, is another important characteristic of this tissue. The ability of the tissues grown in vitro to synthesize their characteristic chemicals was not examined.

Growth and chlorophyll production on modified basic media with or without added sucrose

The growth and chlorophyll contents of cultures from carrot root, endive embryo, lettuce leaf, lettuce stem, parsley stem and spinach leaf were examined on modified basic media with and without sugar.

In the absence of sugar, casein hydrolysate did not support good growth. Callus tissues grown on media containing coconut milk and kinetin had more chlorophyll than those grown on other media without these substances. Spinash tissue had as high as 73 gamma chlorophyll/gram wet weight of tissue. Coconut milk was a more efficient carbon source for growth than sugar.

Comparisons of tissues grown on media supplemented with ascorbic acid or thiourea showed that the addition of these reductants to the media had differential effects depending on the species. Spinach was little, if any, improved. Addition of ascorbic acid favored growth of lettuce tissue and addition of thiourea stimulated growth of endive tissue. The data suggested that some of these chlorophyllous tissues can grow slowly autotrophically on media free of added sugars or amino acids.

Chlorophyll formation and growth was best in spinach tissue on THS + CH-media with more than 0.25% sucrose, in the light, and this tissue had an expecially dark green color. Next best chlorophyll production on this medium was seen with lettuce leaf tissue. On THS (- sucrose) + CH-medium spinach growth was best. On THS (- S) + CH + ascorbic acid lettuce leaf tissue grew best. On THS (- S) + CH + thiourea endive tissue produced a good green color.

Similar experiments with T (- S) or MS (- S) media + coconut milk and casein hydrolysate showed that carrot and lettuce stem tissue were stimulated by light. Carrot, lettuce leaf and stem and spinach were especially favored by light while endive and parsley showed less growth in light than in darkness.

The growth of tissue on the THS + CH medium supplemented with 0.25% sucrose with or without ascorbic acid and exposed to daylengths of 16, 12 or 8 hours daily showed that 12 hours illumination was favorable for growth of carrot and lettuce leaf and stem tissues.

It is felt that under appropriate nutritional and other environmental conditions tissues can be selected and developed will give good autotrophic growth.

Effect of quality and quantity of light

Different wavelengths of light were tested for their effects on growth and chlorophyll production. Chlorophyll content of tissues grown in the dark was considerably lower than those maintained in light. However, chlorophyll developed quickly in dark grown tissues upon their transfer to light. Growth and chlorophyll synthesis were not related to each other. The highest amount of fresh weight increase was achieved when these were grown in the dark, e.g., lettuce leaf, lettuce stem and tomato. Effect of various wavelengths of light on the growth and chlorophyll content of the tissues was variable. Normally, one would expect high rates of growth and chlorophyll formation in red light. The tissues used here were perhaps not actively photosynthesizing under the nutritional and light conditions in these experiments. The effect of light intensity as compared to the effect of quality of light on the growth of tissues was very marked in endive and carrot tissues. Carrot tissue requires high light intensity for maximum growth while the growth of endive tissue is inhibited at high light intensities.

Tissues of seven of the best growing species were grown under white, blue, green, red and dark conditions in the Brunswick incubator chamber. The growth and chlorophyll development were followed in the various callus tissues growing in the Murashige and Skoog synthetic medium. This medium was used to avoid the unknown substances provided with coconut milk. The cultures were grown for five weeks. The results are summarized in Table 19.

The type of light influenced the amount of growth and chlorophyll content of the tissues. The optimal light condition varied depending on whether growth was measured in terms of wet weight or in terms of dry weight per 100 grams of fresh weight. Three of the tissues had the greatest fresh weight when grown in the dark. It is expected from results with chlorophyllous plant callus generally that the amount of growth of these chlorophyllous tissues may be influenced during these first passage studies by the immediately previous environments of the experimental pieces. Further studies are needed to clarify these and related details. For example, chlorophyllous tissues on media without added sucrose might respond differently to various wavelengths of light than these tissues already being supplied adequate sugar. It might take the tissues time to adapt to the new environments.

Differences in the amount of chlorophyll present also appeared under these different light conditions. Some species had much more chlorophyll than others. Except for rose and lettuce stem, dark grown cultures had much less chlorophyll than cultures grown under different light conditions.

Table 19. Growth and chlorophyll development in edible callus tissue on synthetic medium at different wavelengths of light

Tissue	Data*	LIGHT CONDITION				
		White	Blue 385-575 mμ Peak 470 mμ	Green 450-585 mμ Peak 510 mμ	Red 575-700 mμ Peak 650 mμ	Dark
Lettuce Leaf	F.W.	3.538	2.830	2.910	2.926	4.036
	D.W.%	7.9	7.4	7.4	7.6	6.5
	O.D.	0.13	0.12	0.14	0.12	0.05
Lettuce Stem	F.W.	1.565	1.922	2.192	1.598	3.130
	D.W.%	8.0	5.7	5.8	6.6	6.0
	O.D.	0 08	0.06	0.05	0.07	0.06
Tomato Crown Gall	F.W.	3.901	2.760	2.550	4.941	8.588
	D.W.%	7.0	7.5	7.6	6.4	5.8
	O.D.	0.16	0.17	0.18	0.17	0.04
Parsley Petio-	F.W.	3.585	3.123	2.620	2.929	1.758
	D.W.%	7.7	5.8	6.7	6.0	7.0
	O.D.	0.03	0.03	0.06	0.04	0.02
Carrot Root	F.W.	4.806	3.050	5.578	5.753	2.398
	D.W.%	5.8	6.7	4.9	5.3	7.6
	O.D.	0.16	0.13	0.17	0.14	0.06
Endive Embryo	F.W.	2.348	1.890	2.384	2.388	1.802
	D.W.%	7.5	8.0	7.7	7.6	7.4
	O.D.	0.15	0.08	0.08	0.09	0.05
Rose Stem	F.W.	0.935	0.895	0.938	0.954	0.800
	D.W.%	6.2	5.9	6.5	5.8	6.1
	O.D.	0.05	0.03	0.04	0.03	0.05

*F.W. = Average fresh weight in grams of 4 tissue pieces in one bottle;
D.W.% = Dry weight in grams per 100 grams fresh tissue;
O.D. = Optical density of ethanol chlorophyll extract at 665 mμ.
Original tissue transplants weighed 100 mg.

Details about the quantity of light for optimum chlorophyll production and growth have not been completely established. It was observed, however, that carrot tissue required high light intensity (1250-2000 foot candles) for maximum growth. The endive tissue, on the other hand, was inhibited by high light intensity and grew best with 100-225 foot candles.

Differentiation of roots, stems, leaves and plants from callus

Considerable amount of tissue differentiation and organ formation (roots and shoots with leaves) was observed in endive, parsley and lettuce leaf and stem tissues on certain media. Coconut milk-containing media generally did not favor morphogenesis and organ differentiation.

Chlorophyll containing callus tissue derived from mature embryos of Cichorium endivia Linn. (endive or caserole) of the Compositae has been grown on a completely defined nutrient mediu. The tissue breaks up into a thick suspension of cells and cell groups in a liquid medium on a shaker. Gradually, many small round masses of tissue, designated here as embryoids, are formed which differentiate to form numerous small plantlets having typical curled and fringed green leaves and roots (Table 20).

The inherent ability of the cells of an organism for growth, cell division and differentiation into tissues and organs has long been investigated by biologists. The exact potentialities of cells in these directions are still not clearly understood. In order to grow, divide and differentiate, a cell must possess, apart from the complete genetic information which it already has, all the necessary chemical machinery for this purpose or be supplied this and a suitable physical environment. The totipotency of cells has long been accepted mostly on the basis of evidence coming from lower plants and animals. Failure to grow isolated cells from higher plants and animals into mature adult organisms may be due to deficiencies in the necessary chemical imbalances in nutritive requirements and the exact physical conditions needed for such development.

Parsley tissue produced plants by a different route. When the callus was grown on T-medium in the dark it produced many roots. Attached to the roots were embryoids which either, while still attached or when detached, grew and differentiated to produce plantlets (Table 20).

These and related studies further emphasize the potentialities for higher plant callus for food and fiber for human needs. The information already available indicates that these in vitro cultures of plant materials can be induced to differentiate callus from many sources, that callus may produce leaves, stems and roots, and finally, if need be, the differentiated parts can again be induced to produce callus. Thus, cultures can be maintained in various states as desired and when needed can be induced to grow or differentiate in various directions.

These built in potentials suggest the cultures may also be exploited by selection for many other desirable qualities that may be needed.

Table 20. Growth and differentiation in endive embryo callus (EE) and parsley petiole callus (PAR) tissues grown in light and dark in Murashige and Skoog medium with or without various concentrations of indoleacetic acid (IAA) and/or kinetin (KIN). All cultures incubated at 25±2°C for 5 weeks. (Light - continuous illumination at 200 f.c.; dark - dark with occasional short periods of very low intensity light.) Figures indicate fresh weight of tissue in one bottle. Results are average of 32 pieces from 8 bottles.

Medium		Tissues			
IAA	KIN	PAR		EE	
mg/l	mg/l	Light	Dark	Light	Dark
0	0	1.035	0.982**	3.575	1.230
1	0	0.919	0.933**	2.461	1.557
2.5	0	1.100	1.251**	1.484	1.679
5	0	1.076	1.093**	1.088	1.304
10	0	0.915	0.920	0.979#	1.365
0	0.02	2.38**	2.060***	3.492##	1.395##
1	0.02	1.131	1.421**	2.399#	1.740
2.5	0.02	1.057	1.023	2.967#	1.944
5	0.02	2.060	2.016	1.658#	1.630
10	0.02	2.300	1.801	1.203#	1.843
0	0.04	4.675*	6.659****	4.222###	1.817##
1	0.04	3.614*	4.708***	3.934##	2.038
2.5	0.04	1.882*	3.735**	3.663##	2.173
5	0.04	1.519*	3.213**	3.172##	1.766
10	0.04	1.172*	2.178**	2.203##	1.198
0	0.08	1.055*	1.031**	5.170#	2.244
1	0.08	0.978	0.981**	6.000	2.893
2.5	0.08	1.103*	1.050**	3.271	1.768
5	0.08	1.035*	1.343*	1.643	1.658
10	0.08	1.183	1.128*	0.709	1.385
0	1.0	1.569*	2.605***	5.595#	3.143
1	1.0	1.351*	0.935**	6.203#	3.748
2.5	1.0	1.004*	1.300*	5.126#	3.483
5	1.0	1.144*	1.225*	1.733	1.532
10	1.0	1.092	1.223*	0.823	1.265

* - rare to no roots; ** - many roots; *** - many well formed roots; **** - profuse formation of good roots. Most roots negatively geotrophic.
# - small shoots with leaves: ## - well formed shoots with leaves; ### - shoots 0.5" to 1.0" in height with well formed leaves. In light, the shoots and leaves are bright green in color while in cultures maintained in dark the leaves are chlorotic; the same turn green in color when transferred to light. In almost all cases endive tissue differentiated small moss-like leaves.

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13 ABSTRACT

There appears a great potential in tissue cultures of higher plants as a means of producing an abundant supply of fresh, edible, tasty, nutritious plant food for gas exchange in difficult situations and in space travel. Chlorophyllous and nonchlorophyllous strains of edible plant tissues have already been established from many plant species. The requirements for growth and chlorophyll production are influenced by the composition of the medium on which they are grown and by other environmental factors, including light, temperature and acidity of the medium. Nitrate is an excellent source of nitrogen. Tissues grown in liquid media on a shaker or in aerated media tend to fragment into single cells and small clumps of cells. Tissues on agar media may be grown as undifferentiated masses of cells or may be induced to differentiate roots, stems, leaves and plants by modifying the nutrient and other environments. Under space conditions the chlorophyllous tissues would have unlimited sunlight as energy for photosynthesis, would utilize carbon dioxide, and would produce oxygen in the process of synthesizing carbohydrate for food. Such abilities for growth and differentiation as a single cell or as tissue masses and even plants suggest this method has a great built-in potential to select for almost any type of food quality desired.

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Photosynthesizing	0		0			
Food	4		2		4	
Oxygen	4		2		4	
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